Erythropoietin in cardiac ischemia

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Chapter 5

Erythropoietin attenuates heart failure in a hematocrit independent manner

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Abstract

Objective: The hematopoietic hormone erythropoietin (EPO) induces postnatal neovascularization and stimulates endothelial progenitor cells (EPCs). We hypothesized that EPO mobilizes EPCs, increases capillarization and preserves cardiac function in post-myocardial infarction (MI) heart failure, independent of erythropoietic effects.

Methods and Results: Rats underwent coronary ligation and were left untreated (MI group) or long-acting EPO analogue darbepoetin treatment was started 3 weeks after MI in a low-dose (0.4 μg/kg/3 weeks, MI-EPO-low) or high-dose (40 μg/kg/3 weeks, MI-EPO-high). Hematocrit increased in the MI-EPO-high, but not in MI-EPO-low group ($p<0.01$). In both EPO-treated groups, serial echocardiography demonstrated preservation of left ventricular systolic function, and hemodynamic measurements revealed improved cardiac contractility (dP/dt_{max}) and relaxation (dP/dt_{min}) at 9-weeks, compared to MI group (all $p<0.05$). In addition, in EPO-treated groups the number of EPCs was significantly increased (MI-EPO-high: 82 [66-147] and MI-EPO-low: 67 [55-107] vs. MI: 23 [19-33] cells/high-power field; both $p<0.01$). This was associated with a 42% increase in capillary-to-myocyte ratio in MI-EPO-high ($p<0.01$) and 28% in MI-EPO-low ($p<0.05$), compared to MI group.

Conclusions: EPO treatment preserves cardiac function in post-MI heart failure, even in non-erythropoietic doses. This is associated with EPCs mobilization and induction of neovascularization.

Introduction

The classical role of erythropoietin (EPO) is related to its hematopoietic effects. EPO is produced in kidneys and acts as a major regulator of erythropoiesis, by increasing survival and promoting proliferation of erythroid precursor cells.

Recently, several non-erythropoietic effects of EPO have been reported. EPO was shown to render vascular protection in various experimental models of ischemia, including stroke and myocardial infarction ([1]). During *ex-vivo* ischemia-reperfusion in isolated rat hearts, EPO perfusion improved cardiac function and increased coronary flow ([2,3]). Acute, systemic treatment with EPO, in a rodent ischemia-reperfusion model, substantially reduced infarct size and decreased myocardial apoptosis ([4]), even when EPO was administered after reperfusion ([5]).

In addition, EPO was shown to promote postnatal neovascularization, at least in part by enhancing endothelial progenitor cells (EPCs) mobilization from the bone marrow ([6]). EPCs-mediated neovascularization of the peri-infarct zone prevents ventricular remodeling and improves cardiac function ([7]). Interestingly, the level of EPCs predicts cardiovascular outcomes in patients with coronary artery disease ([8]).

Recently, we assessed the effects of EPO treatment in a rat model of post-myocardial infarction (MI) heart failure. In this study, prolonged high-dose EPO treatment was associated with improved cardiac function and neovascularization ([9]).

However, because high-dose EPO treatment is also associated with increased hematocrit values, the observed beneficial effects may, at least to some extend, be related to the increased oxygen-carrying capacity of blood. In the clinical situation, repeated therapy with high-dose EPO could lead to unwanted elevation of hematocrit, coupled with higher risk for thrombo-

sis and hypertension. In addition, the existence of different receptors that mediate the effects of EPO in distinct tissues ([10]), may suggest also different dose-response relationships for the various protective effects.
Therefore, we studied the effects of high- and low-dose EPO treatment on cardiac function over time, EPCs mobilization and neovascularization in a post-MI heart failure.

Methods

Animals

We used male Sprague Dawley rats weighing 270-330 g (Harlan, Zeist, The Netherlands). Animals were fed ad libitum, and housed in groups of four to five rats, according to institutional rules with 12:12 hours light-dark cycles. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Design of the study

Rats were either subjected to left coronary artery ligation (n=63) or sham surgery (n=11). Rats with MI were allocated to 3 groups: control (MI) and two EPO treatment groups with different dosages of long-acting EPO analogue darbepoetin: 40 μg/kg (MI-EPO-high) and 0.4 μg/kg (MI-EPO-low). Darbepoetin-alpha (Aranesp, Amgen Inc., Thousands Oaks, CA, USA) was administered intraperitoneally, once every three weeks, starting three weeks after the coronary artery ligation, hence after the healing phase of MI. Control (MI) and SHAM rats received corresponding injections of saline. The allocation of MI rats to different groups was based on echocardiography performed at week 3 (before the start of the therapy), groups were matched based on left ventricular (LV) end-diastolic diameter and LV fractional shortening.
The high dose of darbepoetin was based on our previous study ([9]), demonstrating increased neovascularization in this model, together with significant elevation of hematocrit levels. To avoid the effect of EPO treatment on hematocrit we included a low-dose EPO group, with 100-times lower darbepoetin dosage (0.4 μg/kg/3 weeks), which in our pilot experiment did not cause elevation of hematocrit (data not shown). Hematocrit was measured at baseline and at week 3, 4, 6 and 9 after surgery.

Myocardial infarction model

This model has been described previously ([11]). Briefly, rats were anesthetized with 2.5% isoflurane and placed on a heating pad (37^{0}C). Animals were intubated and mechanically ventilated (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands; frequency 90/min) using room air enriched with 1.0 l/min oxygen. After left-side thoracotomy, MI was induced by ligating the proximal portion of the left coronary artery, beneath the left atrial appendage. In sham operated rats, the same surgery was performed, without ligating the suture.

Echocardiographic measurements

Cardiac function was assessed by echocardiography (Vivid 7, GE Healthcare, Chalfont St. Giles, United Kingdom; equipped with a 10-Mhz phase array linear transducer) at baseline

(before coronary artery ligation), at week 3 (before start of the therapy), 6 and 8. The echocardiographic measurements were performed under general anesthesia with 2.5% isoflurane, by two researchers blinded for the treatment allocation. The transducer was applied parasternally and maneuvered to obtain both 2-dimensional (2D) images in parasternal long-axis and short-axis view and 2-D guided M-mode tracing. Long axis views were obtained, ensuring that the mitral and aortic valves and the apex were visualized. Short axis views were recorded at the level of mid-papillary muscles. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from the M-mode and calculated as an average from short- and long-axis view. LV fractional shortening (FS %) was calculated as FS= (LVEDD-LVESD)/ LVEDD x 100. LV ejection fraction (EF %) was calculated using the Teichholz method of estimated LV volumes ([12]).

Hemodynamic measurements

After nine weeks rats were anesthetized as described above. Microtip pressure transducer (Millar Instr. Inc., Houston, Texas, USA) was inserted into the left ventricular cavity via the right carotid artery. After a 3-min period of stabilization, heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and developed left ventricular pressure (dLVP=LVSP-LVEDP) were measured. As indices of contractility and relaxation, the maximal rates of increase and decrease in LVP (dP/dt_{max} and dP/dt_{min}) were determined. The catheter was retracted into the aortic arch and arterial systolic and diastolic blood pressures (SBP, DBP) were recorded.

Infarct size and myocyte hypertrophy

After hemodynamic measurements, hearts were rapidly excised and weighed. Mid-papillary slices were fixed in 4% paraformaldehyde and paraffin-embedded. Infarct size was determined by planimeter in transverse slices on picrosirius red/fast green–stained sections. Infarct size was expressed as the percentage of scar length to total left ventricular circumference, as described previously ([13]). Deparaffinized 5-µm thick sections were stained with a Gomori's silver staining. Using image analysis (Zeiss KS 400, Jena, Germany), concentric myocyte hypertrophy in the viable LV wall, remote from the infarcted area, was measured as the cross-sectional area of transversally cut myocytes showing a nucleus ([11]). Myocyte density was calculated as the average number of myocytes per tissue area (mm^2). In each stained section, measurements were averaged from three different counting fields (± 75 myocytes per heart).

Blood derived endothelial progenitor cells

Full blood was collected in heparine tubes (17 IU/ml). Mononuclear cells were isolated using Histopaque-1083 (Sigma Chemical, St. Louis, MO, USA) according to the instructions as supplied by the manufacturer and counted on microcellcounter (Sysmex F-800, Toa Medical Electronics, Kobe, Japan). Isolated mononuclear cells (1×10^6) were seeded into fibronectin-precoated 24-well plates (BD BioCoat, Bedford, MA, USA) in EndoCult medium (StemCell Technologies, London, UK) supplemented with Penicillin (100 U/mL) and Streptomycin (100 µg/mL). After 6 days, adherent cells were washed with medium, incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL (10 µg/mL DiT AcLDL; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 12 hours, fixed with 1% paraformaldehyde for 10 minutes, and counterstained with fluorescein isothyiocyanate-labeled

Table 1. Characteristics of the experimental groups

	Sham	MI	MI-EPO-high	MI-EPO-low
General				
n	11	10	11	9
Infarct size	-	45±3	47±2	50±3
Hemodynamics				
Heart rate (bpm)	321±6	318±8	339±7	312±7
LVSP (mmHg)	130±3	104±6†	119±3*§	115±4†
LVEDP (mmHg)	10±1	24±4†	16±2‡	19±2*
SBP (mmHg)	127±3	102±5†	115±3*‡	113±4*
DBP (mmHg)	81±2	73±3*	83±3§	80±2
Body/organ weight				
BW (g)	410±6	402±12	416±8	442±8*§
Heart weight/BW (mg/g)	4.5±0.1	6.0±0.3†	5.3±0.1†‡	5.3±0.3*‡
Hematocrit				
Baseline (%)	48±0.9	46±0.7	48±0.6	48±0.7
Week 3 (%)	49±0.8	50±0.7	49±0.4	50±0.5
Week 4 (%)	51±0.9	51±0.8	64±1.1†§	52±0.7
Week 6 (%)	49±0.8	50±1.0	56±0.6†§	51±0.5
Week 9 (%)	44±0.6	44±0.6	53±1.4†§	42±0.9

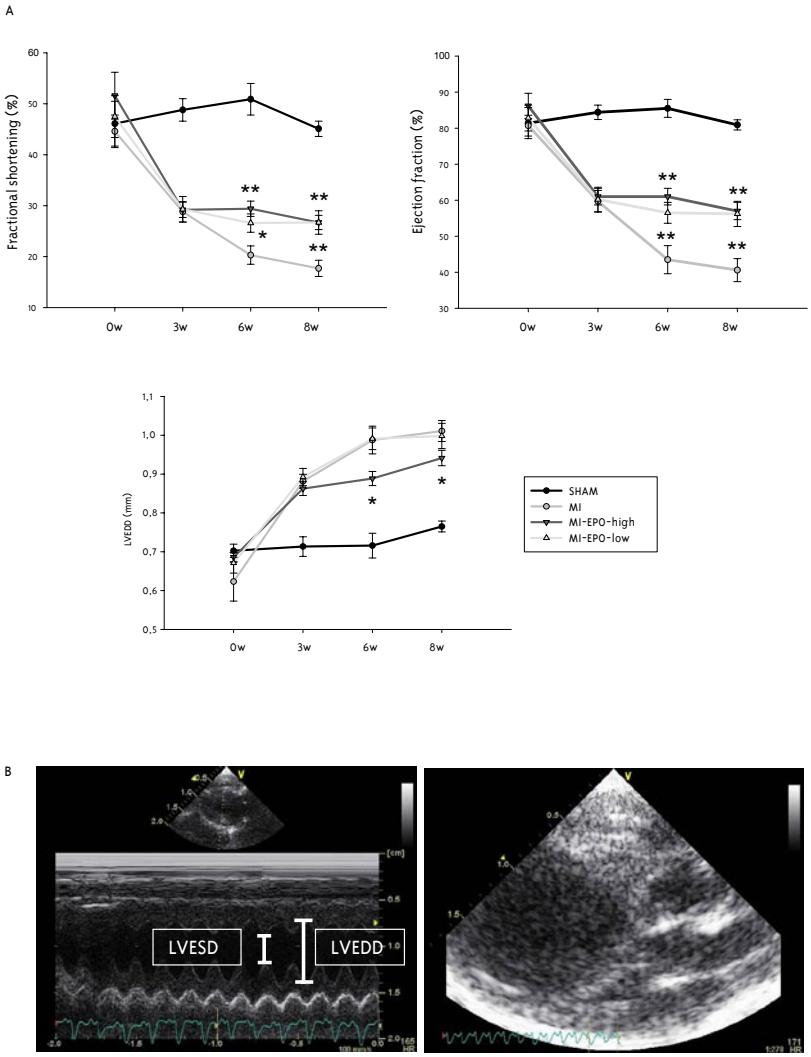
Data are presented as mean ± SEM; n indicates number of animals. bpm, beats per minute; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; BW, bodyweight. *p<0.05; †p<0.01 vs. Sham; ‡p<0.05, §p<0.01 vs. MI.

Griffonia (bandeiraea) simplicifolia lectin I, isolectin B_4 (lectin, 10 µg/mL; Vector Laboratories, Burlingame, CA, USA). Images were captured by an inverted fluorescence microscope (Axiovert 135 M, Carl Zeiss, Jena, Germany). Cells double positive for DiI AcLDL and lectin staining were considered EPCs and counted in high-power fields with the co-localization analysis (Image-Pro Plus for Windows, version 4.5.0.29). For every rat an average number of EPCs was calculated from 4-5 high-power fields.

Capillary density

To visualize the capillaries in the myocardium of the LV free wall, endothelial cells were stained with biotin-labeled Lectin GSL (1:100; Sigma-Aldrich, St. Louis, Missouri, USA), as previously described ([11]). Since lectins stain not only capillaries but other vessels as well, a size criterion of 10 µm was used to exclude small arterioles and venules. Image analysis (Image-Pro Plus for Windows, version 4.5.0.29) was used to measure capillary density, calculated as the number of capillaries per tissue area (mm^2). As a measure of neovascularization, capillary-to-myocyte ratio was calculated dividing capillary with myocyte density, as previously described ([11]).

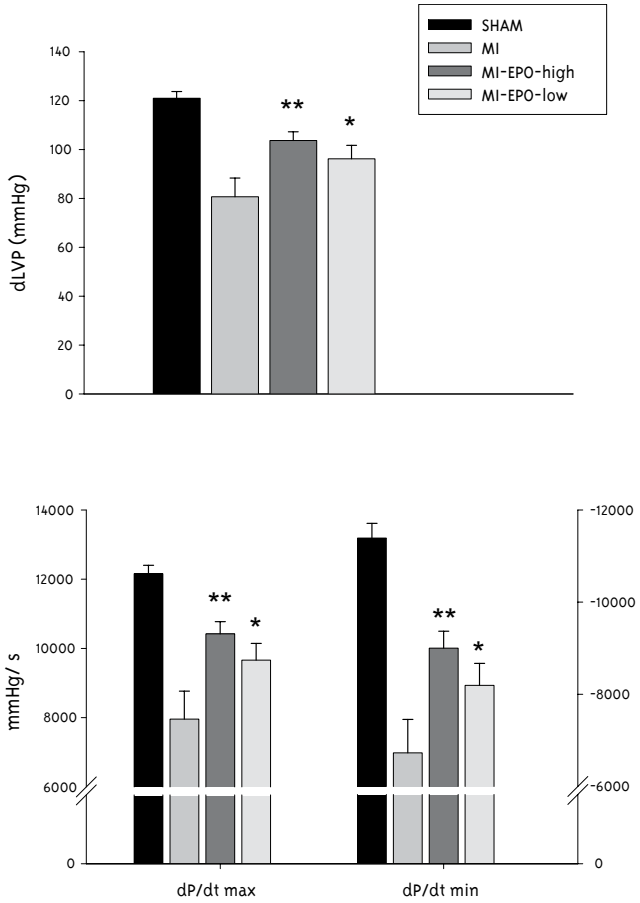
Figure 1. A, Changes in echocardiographic indices of LV size and function during 8-week follow-up after coronary artery ligation. * p<0.05 vs. MI, ** p<0.01 vs. MI. B, M-mode echocardiographic picture of a sham rat with calculation of LV size (left picture); 2D-picture of long axis in a rat after MI (right picture); LVEDD indicates left ventricular end-diastolic diameter; LVESD left ventricular end-systolic diameter.



Statistical analysis

Data are presented as mean ± SEM, or as median ± IQR (25^{th} and 75^{th} percentile) depending on their distribution. Differences among groups were tested using one-way analysis of variance, followed by LSD post-hoc analysis if normally distributed, and by Kruskal-Wallis test if skewed distributed. Correlation analysis was performed with Spearman's correlation test. All reported probability values were 2-tailed, and a p-value <0.05 was considered statistically significant.

Figure 2. Effects of myocardial infarction and different doses of EPO treatment on hemodynamic parameters. dLVP indicates developed left ventricular pressure; dP/dtmax and dP/dtmin, maximal rate of increase and decrease of ventricular pressure, respectively. * p<0.05 vs. MI, ** p<0.01 vs. MI.



Results

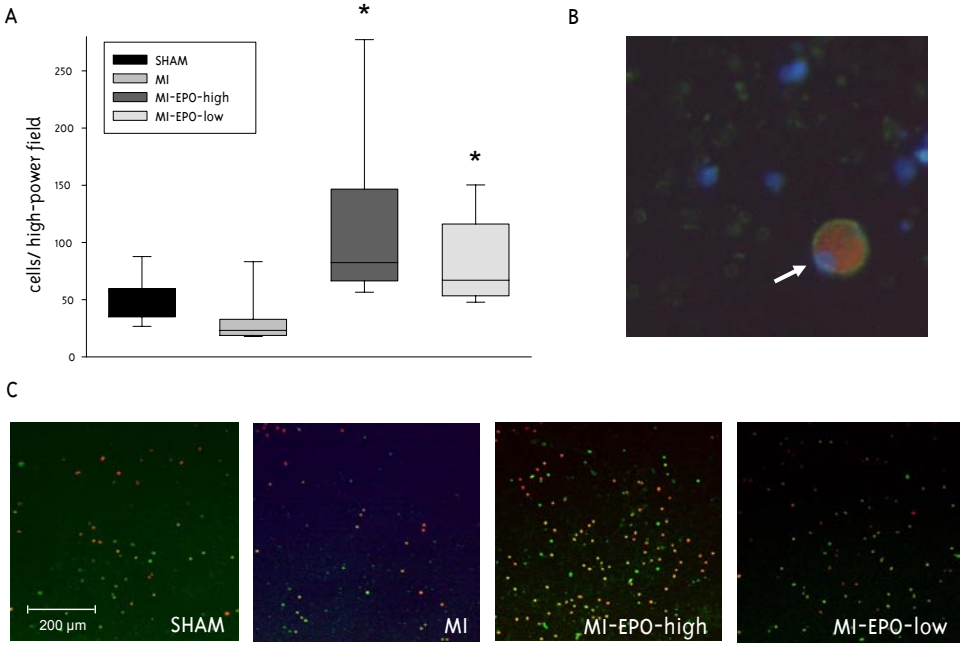
Mortality and general characteristics

Overall 24-hour mortality following the MI was 41%. Five additional MI rats died during the 9-week long follow-up (2 in MI group, 1 in MI-EPO-high and 2 in MI-EPO-low group).

General characteristics after nine weeks are shown in table 1. Two rats (1 in MI and 1 in MI-EPO-high group) had infarct size < 25%. These were excluded from further analysis. LV-infarct size (% of LV) was comparable between all MI groups (table 1). Body weight (BW) was significantly higher only in the MI-EPO-low group (table 1).

The heart weight to BW ratio was significantly increased in the rats with MI compared to the sham rats (all $p<0.05$; table 1). A lower heart weight to BW compared to MI group was observed in MI-EPO-high and MI-EPO-low groups (both $p<0.05$).

Figure 3. Effect of EPO treatment on number of circulating EPCs. A, Graphic representation of number of EPCs. The boxplots show the median with 25-75% range, the error bars show 10-90% range of the number of EPCs per high-power field. *p<0.01 vs. MI. B, Endothelial progenitor cell under high magnification (white arrow), positively stained for DiI AcLDL (red cytoplasm) and lectin (green membrane), including DAPI nuclear staining (blue). C, Representative microscopic fields from all experimental groups, with double stained (green+red) positive EPCs.



Low-dose EPO does not increase hematocrit

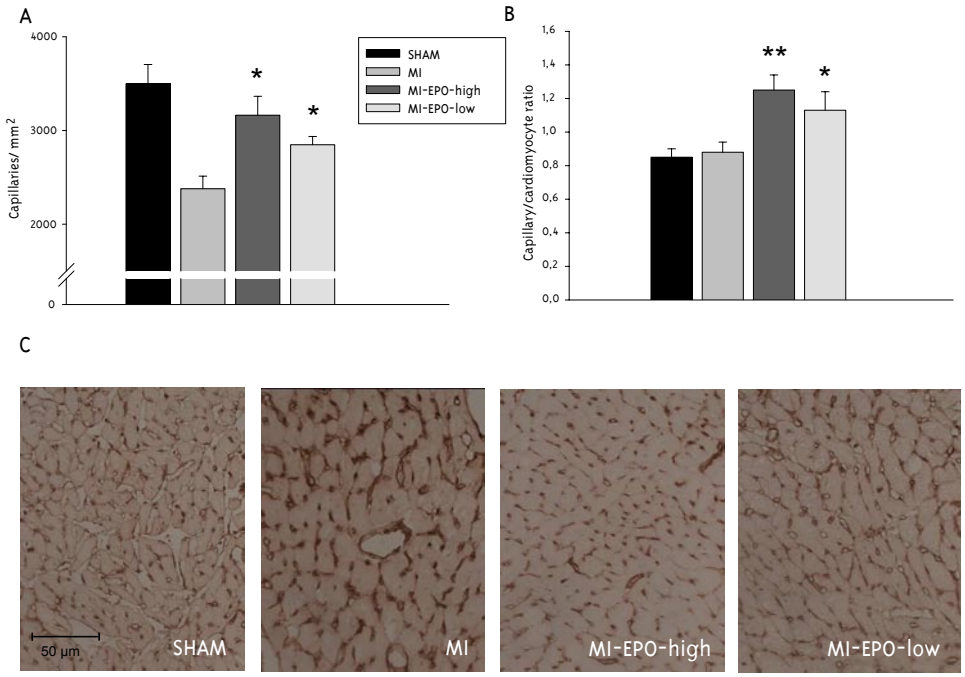
The changes of the hematocrit throughout the experiment are shown in table 1. Only the treatment with high-dose EPO led to significant increase in hematocrit levels, which persisted throughout the experiment. Importantly, hematocrit levels in MI-EPO-low group were similar to those of MI and sham groups.

Low-dose EPO improves cardiac function

Serial echocardiographic parameters are presented in figure 1. Baseline echocardiographic measurements of the LV size and function did not differ among groups before the coronary artery ligation. Induction of MI led to a significant enlargement of LVEDD and deterioration of LV performance (FS and EF) at 3 weeks. While the deterioration of LV performance progressed gradually in the MI group throughout the remaining 6 weeks, it remained stable after the initiation of EPO treatment in both the MI-EPO-high and MI-EPO-low group. Treatment with high-dose EPO prevented the progression of LV dilation (LVEDD), when compared to MI group at week 6 and 8 (both $p<0.05$). In contrast, low-dose EPO treatment did not decelerate the LV dilation after the MI.

To further evaluate the hemodynamic profile of all experimental groups, invasive pressure measurements were performed 9 weeks after the surgery, directly before the rats were

Figure 4. Effect of EPO treatment on neovascularization. A, Actual measurements of capillary density in number of capillaries per mm^2. B, Bar graph representing the capillary-to-myocyte ratio in different groups. C, Tissue sections with lectin in the viable free wall of the four different groups, showing individual capillaries. *p<0.05 vs. MI, ** p<0.01 vs. MI



sacrificed. Myocardial contractility (dP/dt_{max}) and myocardial relaxation (dP/dt_{min}) were both impaired in all MI groups compared to the sham group (all $p<0.05$). Both low- and high-dose EPO treatments resulted in better contractility and relaxation compared to MI (all $p<0.05$; figure 2). LVSP and developed LVP (dLVP) were both decreased in all MI groups compared to sham operated rats ($p<0.05$ for all). MI-EPO-high showed a significantly higher LVSP (table 1) and dLVP (figure 2), compared to MI (both $p<0.01$). Low-dose of EPO resulted in a 17% higher dLVP ($p<0.05$; figure 2), and a trend towards elevation of LVSP, compared to control group ($p=0.07$; table 1). LVEDP was elevated in MI-group compared to sham operated rats ($p<0.01$; table 1). In the MI-EPO-high group the LVEDP was 34% ($p<0.05$) and in the MI-EPO-low group 20% (p=NS) lower, compared to MI. The SBP and DBP were higher only in the MI-EPO-high group, compared to control (both $p<0.05$).

EPO dose-dependently mobilizes endothelial progenitor cells

Induction of MI led to a decrease in the number of EPCs, at 9 weeks ($p<0.05$; figure 3). Treatment with both high- and low-dose EPO resulted in a 3.6- and 2.9-fold increase in the number of EPCs, respectively, compared to MI group (both $p<0.01$; figure 3).

Low- and high-dose EPO treatment induces neovascularization

Figure 4C shows representative photomicrographs of the four different groups. Capillary density was significantly reduced in MI compared to sham-group ($p<0.01$). High-dose EPO treatment prevented the decrease in capillary density after induction of MI and restored it to sham values, as shown in Figure 4A (p=NS vs. sham). In this group (MI-EPO-high) we observed a 33% increase in capillary density compared to MI group ($p<0.01$). Treatment with low-dose EPO resulted in a 20% higher capillary density ($p<0.05$). The cross-sectional area of cardiomyocytes increased in MI compared to sham group (290 ± 16 vs. 204 ± 10 μm^2; $p<0.01$). EPO treatment had no effect on attenuation of myocyte hypertrophy (MI-EPO-high: 286 ± 15 and MI-EPO-low: 323 ± 27 μm^2). However, the capillary-to-myocyte ratio increased by 42% in MI-EPO-high ($p<0.01$) and by 28% in MI-EPO-low ($p<0.05$), compared to MI group (figure 4B). The differences between MI-EPO-high and MI-EPO-low group were not statistically significant.

In order to relate mobilization of EPCs to increased capillary-to-myocyte ratio, correlations were determined. We observed a positive correlation between the capillary-to-myocyte ratio and the number of peripheral EPCs ($r=0.48$, $p<0.01$), which was even stronger in the rats subjected to coronary artery ligation ($r=0.60$, $p<0.01$).

Discussion

In the present study, we demonstrate beneficial effects of EPO on cardiac function in a post-MI heart failure model, without affecting hematocrit. Furthermore, this is associated with mobilization of EPCs and increased capillary-to-myocyte ratio, indicating new vascular growth.

Recently, several experimental studies have demonstrated important ancillary functions of EPO, such as protection against ischemic injury in various tissues. In the first in vivo study on the EPO effects in the heart, Calvillo *et al.* ([14]), employed a rat model of coronary ischemia-reperfusion. Administration of EPO (5.000 IU/kg/day) for seven consecutive days after reperfusion reduced the loss of cardiomyocytes by 50%, an extent sufficient to normalize hemodynamic function. However, the hematocrit increased by 20-30% by the end of the study, and to some degree, such a change could lead to improved cardiac function merely by improving the delivery of oxygen.

In the clinical setting, current therapy in patients after MI is focused on prevention of ventricular remodeling and development of heart failure. Myocardial regeneration may offer possibilities that could improve cardiac function in these patients ([15]). Although cardiomyocytes proliferation after ischemic injury seems limited, the formation of new vessels in the non-infarcted part of the ventricle could lead to an improvement of function and attenuation of ventricular remodeling ([16,17]).

EPO was recently shown to mobilize EPCs from the bone marrow, which was associated with neovascularization (vasculogenesis) of ischemic tissue ([6]). EPO has also been shown to stimulate proliferation of endothelial cells in situ (angiogenesis) ([18]). The effect of EPO on the formation of new vessels has been observed in an experimental model of stroke. EPO treatment, initiated 24 hours after induction of stroke, enhanced neovascularization and improved neurological function, whereas it did not significantly influence infarct size([19]). In this study, high-dose EPO treatment increased the density of microvessels at the stroke

boundary (ischemic penumbra), but it also resulted in a 44% increase in hematocrit level.
We addressed this issue in the heart, evaluating the effect of EPO treatment on new vascular formation in an experimental heart failure model ([9]). Rats were subjected to coronary artery ligation and therapy with high-dose EPO analogue darbepoetin was initiated 3-weeks post-MI. Although not reducing infarct size, EPO treatment significantly improved cardiac function. This improvement was coupled to increased capillary density and capillary-to-myocyte ratio, indicating neovascularization. Furthermore, these beneficial effects were also associated with increased percentage of alpha-MHC (myosine-heavy chain) isoforms, a molecular marker of enhanced myocardial contractility.
However, the dosages used in the previous studies, when applied to clinical situation, could cause EPO overdose which may lead to hypertension, seizures, vascular thrombosis and death, possibly related to abruptly increased hematocrit values ([20]). This could be of potential concern in patients with already elevated cardiovascular risk.
In the present study, we investigated effects of a non-erythropoietic EPO dose to avoid elevation of hematocrit, and consequently the changes in rheology and oxygen-binding capacity of the blood, which could both influence our results. We compared these effects to those of a high EPO dose (MI-EPO-high), leading to marked hematocrit elevation, which effects on cardiac function in post-MI heart failure were already established ([9]). The low-dose of EPO was two orders of magnitude lower compared to high-dose. We did not observe any differences in hematocrit levels between the MI-EPO-low and both saline treated groups (MI and sham), while in MI-EPO-high the hematocrit remained significantly elevated during the treatment follow-up.
Treatment with low-dose EPO prevented the deterioration of LV function over time, as assessed by LV ejection fraction and fractional shortening, serially evaluated by echocardiography. However, low-dose EPO, as opposed to high-dose, did not avoid the progression of LV dilation. While this could mean a dose-dependent effect of EPO, bigger infarct size in MI-EPO-low group, although not significantly different from other groups, could have averted the beneficial effects of EPO on LV geometry. Low-dose EPO treatment thus, in spite of advanced LV dilation, improves contractile properties of the non-infarcted part of the myocardium. Importantly, this effect was also confirmed by invasive hemodynamic measurements at the end of the study. Although the treatment with high-dose resulted in noticeable improved cardiac function, low-dose treatment also caused a significant enhancement of developed LVP, together with contractility and relaxation indices of the LV.
We confirmed the findings of our previous experiments, that high-dose EPO treatment increases capillary-to-myocyte ratio, which is indicative of neovascularization. In addition, low-dose EPO, although to a lesser extent, raised the capillary-to-myocyte ratio by 28%. Both high- and low-dose EPO treatment increased the number of EPCs. This is line with findings of Bahlmann *et al.*([21]), who found that darbepoetin stimulates bone marrow-derived EPCs at doses that do not increase the hematocrit. Low-dose of EPO thus differentially stimulates the bone marrow precursor cells (i.e. erythroid vs. endothelial). Moreover, the increased number of EPCs was significantly correlated with capillary-to-myocyte ratio, providing an interesting link between EPCs mobilization and increased vascular growth in the heart.
Application of lower doses of EPO was also shown to confer vascular and tissue protection in the kidney ([22]). Low-dose darbepoetin treatment in a rat remnant kidney model improved the survival, ameliorated endothelial damage and preserved renal function, without increase in hematocrit levels. Another option to avoid the negative effects of chronic EPO therapy on

hematocrit values, could be the use of recently discovered non-erythropoietic derivates of EPO, retaining the tissue protecting property, without undesired effect on erythropoiesis ([23]). The possibility to separate the erythropoietic and tissue-protective effect could be explained through interaction of EPO with different receptors in bone marrow and in "peripheral" tissues ([24]). However, the role of these receptors in EPCs stimulation is unknown, and thus the regenerative capacity of the novel EPO derivates may be limited by reduced mobilization of EPCs.
In summary, EPO treatment preserves cardiac function in post-MI heart failure, even in doses not increasing hematocrit. This is associated with stimulation of EPCs mobilization and induced neovascularization. Although time-limited treatment with high-dose EPO may be beneficial and safe during acute ischemic injury, if prolonged therapy is required (heart failure), drug regimens using low-dose EPO may be more suitable to avoid the adverse effects of the treatment.

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Reference List

1. Van der Meer P., Voors AA, Lipšic E, van Gilst WH, van Veldhuisen DJ. Erythropoietin in cardiovascular diseases. *Eur Heart J* 2004;25:285-291.

2. Cai Z, Semenza GL. Phosphatidylinositol-3-kinase signaling is required for erythropoietin-mediated acute protection against myocardial ischemia/reperfusion injury. *Circulation* 2004;109:2050-2053.

3. Van der Meer P, Lipšic E, Henning RH, De Boer RA, Suurmeijer AJ, Van Veldhuisen DJ, Van Gilst WH. Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury. *Eur J Heart Fail* 2004;6:853-859.

4. Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, Koch WJ. A novel protective effect of erythropoietin in the infarcted heart. *J Clin Invest* 2003;112:999-1007.

5. Lipšic E, van der Meer P, Henning RH, Suurmeijer AJ, Boddeus KM, Van Veldhuisen DJ, Van Gilst WH, Schoemaker RG. Timing of erythropoietin treatment for cardioprotection in ischemia/reperfusion. *J Cardiovasc Pharmacol* 2004;44:473-479.

6. Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* 2003;102:1340-1346.

7. Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Hanley A, Silver M, Kearney M, Losordo DW, Isner JM, Asahara T. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. *Circulation* 2003;107:461-468.

8. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005;353:999-1007.

9. Van der Meer P, Lipšic E, Henning RH, Boddeus K, van der Velden J, Voors AA, Van Veldhuisen DJ, Van Gilst WH, Schoemaker RG. Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction. *J Am Coll Cardiol* 2005;46:125-133.

10. Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, Latini R, Xie QW, Smart J, Su-Rick CJ, Pobre E, Diaz D, Gomez D, Hand C, Coleman T, Cerami A. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. *Proc Natl Acad Sci U S A* 2004;101:14907-14912.

11. Van Kerckhoven R, van Veghel R, Saxena PR, Schoemaker RG. Pharmacological therapy can increase capillary density in post-infarction remodeled rat hearts. *Cardiovasc Res* 2004;61:620-629.

12. Teichholz LE, Kreulen T, Herman MV, Gorlin R. Problems in echocardiographic volume determinations: echocardiographic-angiographic correlations in the presence of absence of asynergy. *Am J Cardiol* 1976;37:7-11.

13. Westendorp B, Schoemaker RG, Buikema H, Boomsma F, van Veldhuisen DJ, van Gilst WH. Progressive left ventricular hypertrophy after withdrawal of long-term ACE inhibition following experimental myocardial infarction. *Eur J Heart Fail* 2005.

14. Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, Salio M, Cerami A, Brines M. Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. *Proc Natl Acad Sci U S A* 2003;100:4802-4806.

15. Lee MS, Makkar RR. Stem-cell transplantation in myocardial infarction: a status report. *Ann Intern Med* 2004;140:729-737.

16. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 2005;115:572-583.

17. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004;428:664-668.

18. Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F. Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood* 1999;93:2627-2636.

19. Wang Y, Zhang ZG, Wang L, Zhang RL, Chopp M. Erythropoietin enhances neurogenesis and angiogenesis in the brain and improves functional recovery after embolic stroke in the adult rat. *Stroke* 2004;35:239.

20. Besarab A, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto DM, Schwab SJ, Goodkin DA. The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *N Engl J Med* 1998;339:584-590.

21. Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, Boehm SM, Menne J, Haller H, Fliser D. Erythropoietin regulates endothelial progenitor cells. *Blood* 2004;103:921-926.

22. Bahlmann FH, Song R, Boehm SM, Mengel M, von Wasielewski R, Lindschau C, Kirsch T, de Groot K, Laudeley R, Niemczyk E, Guler F, Menne J, Haller H, Fliser D. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persistently activates endothelial Akt and attenuates progressive organ failure. *Circulation* 2004;110:1006-1012.

23. Fiordaliso F, Chimenti S, Staszewsky L, Bai A, Carlo E, Cuccovillo I, Doni M, Mengozzi M, Tonelli R, Ghezzi P, Coleman T, Brines M, Cerami A, Latini R. A nonerythropoietic derivative of erythropoietin protects the myocardium from ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* 2005;102:2046-2051.

24. Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M, Savino C, Bianchi M, Nielsen J, Gerwien J, Kallunki P, Larsen AK, Helboe L, Christensen S, Pedersen LO, Nielsen M, Torup L, Sager T, Sfacteria A, Erbayraktar S, Erbayraktar Z, Gokmen N, Yilmaz O, Cerami-Hand C, Xie QW, Coleman T, Cerami A, Brines M. Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 2004;305:239-242.